

## **EXHIBIT 6**

ORIGINAL ARTICLE

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## Antitumor efficacy of regional oncolytic viral therapy for peritoneally disseminated cancer

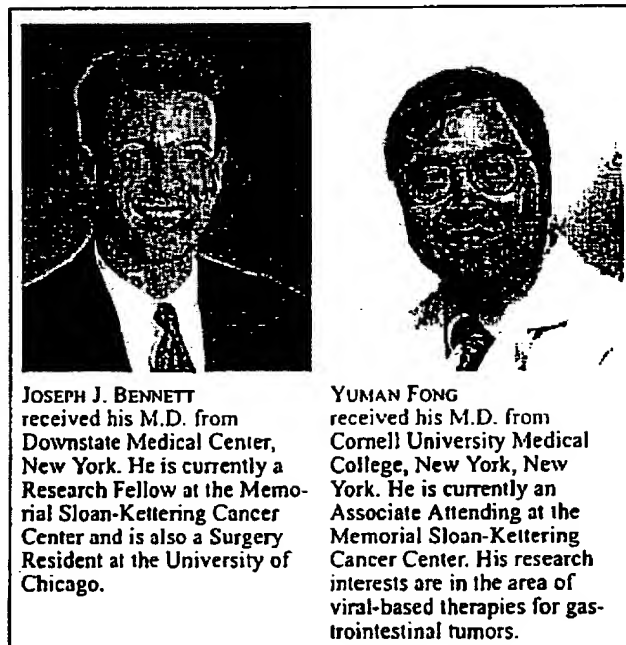
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**Abstract** Oncolytic viral therapy is a promising new method of cancer treatment. Peritoneal dissemination of cancer is a common and fatal clinical condition seen in many malignancies, with few effective therapies avail-

able. G207, a multimutated replication-competent herpes simplex virus type-1, effectively treats disseminated peritoneal cancer. This study evaluates viral proliferation and subsequent tumoricidal effects in vitro and in vivo after regional viral delivery. In vitro studies demonstrate that G207 efficiently kills five human gastric cancer cell lines, and that permissiveness to viral replication is correlated with cytotoxicity. In a murine xenograft model of human gastric carcinomatosis, peritoneal delivery of G207 effectively kills tumor and prolongs survival. Data from quantitative PCR characterizes peritoneal clearance of virus after intraperitoneal injection, and identifies G207 replication within tumor cells in vivo, similar to in vitro proliferation. Further analysis of various organs confirms that G207 does not replicate within normal tissue after peritoneal delivery. Wild-type KOS viral replication was also demonstrated in vivo, with significant toxicity secondary to dissemination and encephalitis. In vivo viral proliferation of G207 is restricted to tumor cells, is correlated with in vitro assays, and is an important mechanism of anticancer efficacy.

**Key words** Peritoneal tumor · Herpes simplex virus · Herpes simplex virus · Stomach cancer · Gene therapy

**Abbreviations** *HSV*: Herpes simplex virus · *MOI*: Multiplicity of infection · *PCR*: Polymerase chain reaction



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### Introduction

Gastric cancer is associated with a high rate of peritoneal dissemination and a 5-year survival of less than 20% [1]. Morbidity and mortality from gastric cancer are usually attributed to intraperitoneal recurrence, which occurs early and often without extra-abdominal metastases [2, 3]. Untreated patients with peritoneal disease have a median survival of 5–10 months [4]. Chemotherapy is the only treatment currently available for carcinomatosis, and provides only a minor survival advantage given either systemically

or intraperitoneally [5, 6, 7, 8]. Active investigation seeks novel effective therapy for this fatal disease process.

Several viral based strategies to treat peritoneal disease are under investigation. Most rely on nonreplicating vectors to transfer therapeutic immunomodulatory genes [9, 10, 11]. Another promising strategy is the use of oncolytic viruses, which have been genetically engineered to decrease virulence and increase specificity for dividing cells, while maintaining direct tumoricidal effects. Herpes simplex virus (HSV) type 1 mutants have been shown to infect and selectively lyse tumor cells while sparing normal tissue. Many were originally designed to treat tumors of the central nervous system, since herpes viruses are naturally neurotropic [12, 13, 14]. There is also increasing evidence that HSV-1 based oncolytic viruses may effectively treat nonneurological malignancies [15, 16, 17, 18]. G207 is a second-generation, replication-conditional, multimutant herpes simplex virus type-1. Based on the wild-type HSV-1 F-strain, G207 contains deletions of both  $\gamma_134.5$  genes, and has an *Escherichia coli lacZ* insertion mutation at the *ICP6* gene [13, 14]. These mutations attenuate neurovirulence and latency and restrict viral replication to tumor cells [19, 20, 21, 22]. The current study evaluates G207 and demonstrates effective killing of human gastric cancer cells in vitro as well as in an animal xenograft model of gastric carcinomatosis.

One major theoretical advantage of replication-competent viruses is their potential to replicate in tumor and exert effects beyond the limited number of viral particles initially administered. While other studies have demonstrated the ability of oncolytic viruses to proliferate in tumor cells in vitro, the current study documents such proliferation in vivo and demonstrates that in vitro viral proliferation assays may predict cytotoxicity and in vivo behavior [12, 13, 18, 23]. These data demonstrate herpes-based oncolytic viral therapy to be a potential treatment of gastric carcinomatosis.

## Materials and methods

### Cells and cell culture

Five human gastric cancer cell lines were used in this study. AGS cells were obtained from the American Type Culture Collection (Rockville, Md., USA) and were maintained in Ham's F-12. MKN-1 and MKN-74 cells were supplied as a generous gift from Dr. Tetsuro Kubota at Keio University, Japan, and were maintained in RPMI 1640. MKN-45-P cells were supplied as a generous gift from Dr. Yutaka Yonemura at Kanazawa University, Japan, and maintained in RPMI 1640. OCUM-2MD3 cells were supplied as a generous gift from Dr. Masakazu Yashiro at Osaka City University Medical School, and were maintained in DMEM with high glucose, 2 mM L-glutamine, and 0.5 mM sodium pyruvate. All media contained 10% fetal calf serum, 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were maintained in a 5% CO<sub>2</sub> humidified incubator at 37°C.

### Virus

G207 is an engineered herpes simplex virus type-1 constructed from R3616 with deletions of both  $\gamma_134.5$  genes, and an insertion

of the *E. coli lacZ* marker gene into the *U<sub>L</sub>39* gene [13]. The  $\gamma_134.5$  deletion decreases neurovirulence of the virus [13, 19, 20]. Disruption of the *U<sub>L</sub>39* gene eliminates ribonucleotide reductase activity and increases specificity of the virus for proliferating cells such as tumor cells [15, 21, 22]. Virus was propagated as previously described and titrated by standard plaque assay [13, 14].

### In vitro cytotoxicity of G207

Five different human gastric cancer cell lines were evaluated for sensitivity to G207 in vitro. Cells were plated at  $5 \times 10^4$  cells/well in 12-well plates (Costar, Corning, Corning, N.Y., USA) and infected 18 h later with multiplicity of infection (MOI) levels of 0, 0.01, 0.1 and 1. Cell viability was determined at 24 h intervals using trypan blue staining.

### In vitro viral proliferation

Viral growth curves were performed to compare the ability of G207 to replicate in different human gastric cancer cell lines. Cells were plated at  $5 \times 10^5$  cells/well in 6-well plates and infected 18 h later with an MOI of 0.01 (5000 pfu). Cells and supernatants were harvested at 6, 24, 48, and 72 h postinfection. Viral titers were determined by standard plaque assay.

### Animal studies

Athymic 4- to 6-week-old male mice purchased from the National Cancer Institute (Bethesda, Md., USA) from pathogen-free stocks were used for all animal experiments. All animal work was approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee and performed under strict guidelines. Animals were anesthetized by methoxyflurane inhalation for experimental procedures.

### Treatment of gastric carcinomatosis with intraperitoneal G207

Intraperitoneal (i.p.) injection of OCUM-2MD3 human gastric cancer cells develops peritoneally disseminated tumors, bloody ascites, and cachexia in an established xenograft model [24]. Fifty athymic mice were inoculated intraperitoneally with  $2 \times 10^6$  OCUM-2MD3 human gastric cancer cells suspended in 1 cc media. Viral treatment ( $n=10$ /group) included i.p. injection of  $5 \times 10^6$  (low dose) or  $5 \times 10^7$  (high dose) plaque-forming units (pfu) of G207, either 3 h or 3 days after tumor challenge. Control animals were treated with i.p. injection of serum-free media. Animals were killed 23 days after initial tumor challenge due to overwhelming tumor burden in the control group. Peritoneal tumor burden was assessed by weight. At this time all abdominal organs with associated peritoneum were removed en bloc. The mesentery, diaphragm, omentum, gonadal fat, and all associated tumor were systematically stripped from the bowel and associated organs and weighed as peritoneal tumor specimens. Organs and carcasses of each animal were weighed. Liver, bowel, spleen, testes, bladder, kidneys, heart, lung, brain, and peritoneal tumor were harvested and frozen in Tissue-Tek embedding medium (Sakura Finetek, Torrance, Calif., USA).

### Survival study after intraperitoneal G207 treatment of gastric carcinomatosis

Mice ( $n=27$ ) were injected i.p. with  $2 \times 10^6$  OCUM-2MD3 human gastric cancer cells suspended in 1 cc media. Viral treatment included ( $n=9$ /group) i.p. injection of  $5 \times 10^7$  pfu of G207 either 3 h or 3 days after tumor challenge. Control mice received i.p. serum free media 3 days after tumor challenge. Animals were checked daily for survival.

### Determination of peritoneal viral clearance and viral proliferation in vivo

Mice were inoculated i.p. with  $2 \times 10^6$  OCUM-2MD3 human gastric cancer cells suspended in 1 cc media. Three days after tumor challenge all animals received i.p. injection of  $5 \times 10^7$  pfu of G207. Animals were divided into groups of three and killed 0, 1, 3, 7, and 12 days after peritoneal delivery of G207. Animals at each time point underwent peritoneal lavage, tumor harvest, and organ harvest. Peritoneal lavage was performed by i.p. injection of 10 cc media followed by aspiration of lavage fluid. Peritoneal tumor, liver, spleen, heart, lungs, brain, testes, bladder, kidneys, and bowel were harvested from each animal as described above. Lavage fluid was frozen as collected. Specimens were divided and both snap-frozen in liquid nitrogen for polymerase chain reaction (PCR) analysis, and frozen in Tissue-Tek medium for histochemical analysis.

### Histochemical analysis of the extent of G207 infection and dissemination in vivo

Histochemical analysis was performed on G207 specimens collected 1, 3, 7, 12, and 20 days after peritoneal delivery of virus. Peritoneum with tumor, liver, spleen, heart, lungs, brain, testes, bladder, kidneys, and bowel were all sectioned, fixed, and evaluated for  $\beta$ -galactosidase (*lacZ*) expression by staining with X-gal solution (Sigma, St. Louis, Mo., USA). Slides were counterstained with nuclear fast red (Sigma).

### Quantification of peritoneal viral clearance, viral dissemination, and viral proliferation of G207 and wild-type KOS virus in vivo

Real-time quantitative PCR was performed on genomic DNA extracted from specimens collected from animals treated with either G207 or KOS virus, with amplification of the herpes *ICP0* immediate-early gene. To evaluate wild-type virus several animals inoculated with OCUM-2MD3 cells were infected i.p. with  $5 \times 10^7$  pfu of KOS virus. Mechanical disruption and phenol/chloroform genomic DNA extraction was performed on tumor specimens collected 0, 1, 2, 3, 5, 7, 12, 20, and 100 days post-G207 delivery. KOS treated animal tumor and brain tissues were evaluated at the same times until day 7, at which point the mice were killed secondary to viral dissemination and encephalitis. Organ specimens and lavage fluid collected 0, 1, 3, 7, 12, and 20 days post-G207 infection were also extracted for genomic DNA. Following phenol/chloroform extraction on lavage specimens yeast tRNA was added as a carrier prior to precipitation. Standard curves were established by spiking uninfected specimens with known quantities of either G207 or KOS virus and performing parallel DNA extractions with the unknown samples. PCR was performed on 1  $\mu$ g genomic DNA extracted from organ and tumor specimens, and on genomic DNA extracted from 250  $\mu$ l lavage fluid. The ABI Prism 7700 Sequence Detector (PE Applied Biosystems) was used to perform real-time quantitative PCR. Forward (5'-ATGTTTCCC-GTCTGGTCCAC-3') and reverse (5'-CCCTGTGCGCTTACGT-GAA-3') primers, and a dual labeled fluorescent TaqMan probe

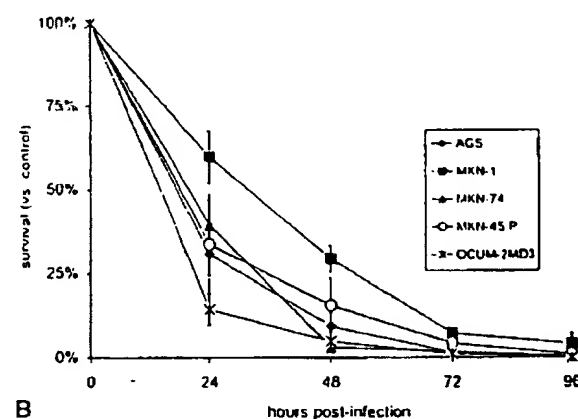
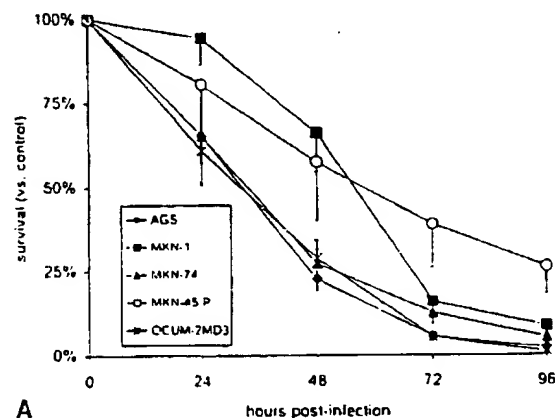


Fig. 1A, B In vitro cytotoxicity of G207. Monolayer cultures of each of five human gastric cancer cell lines were infected at MOIs of 0.1 (A) or 1 (B). Cells were counted every 24 h by trypan blue exclusion.

(5'-6FAM-CCCCGTCTCCATGTCCAGGATGG-TAMRA-3') were designed to amplify a 111-bp fragment of the herpes *ICP0* immediate-early gene. To further normalize the amount of total DNA in each sample the 187-bp coding sequence for 18S rRNA was amplified using forward (5'-CGCCTACCACATCCAAGGAA-3') and reverse (5'-GCTGGAATTACCGCGGCT-3') primers and the dual labeled TaqMan probe (5'-VIC-TGCTGGCACCAGCTTGCCCTC-TAMRA-3'). Samples were subjected to 40 cycles of PCR (stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 s and 60°C for 1 min; and stage 4, 25°C soak).

## Results

### In vitro cytotoxicity of G207

The ability of G207 to kill five human gastric cancer cell lines was demonstrated in vitro at MOI levels of 0.01, 0.1, and 1 (Table 1, Fig. 1A, B). At an MOI of 1, 96-h cell death by G207 was 100% in three cell lines (OCUM-2MD3, AGS, and MKN-74) and 96–99% in two cell lines (MKN-1 and MKN-45-P). An MOI of 0.1 killed 95–99% of cells in three cell lines (OCUM-2MD3, AGS and MKN-74), with 74–91% cytotoxicity observed in the other two cell lines by 96 h (MKN-1 and MKN-45-P). More modest cytotoxicity was seen at the lowest

Table 1 Relationship between viral burst and cell kill in vitro after G207 infection at an MOI=0.01 (5000 pfu)

Cell line	Viral burst at 48 h ( $\times 10^3$ pfu)	Viral burst at 72 h ( $\times 10^3$ pfu)	Cell kill at 96 h
MKN-74	820	790	87%
OCUM-2MD3	38	117	77%
AGS	29	51	65%
MKN-1	14	36	48%
MKN-45-P	3.6	10	9%

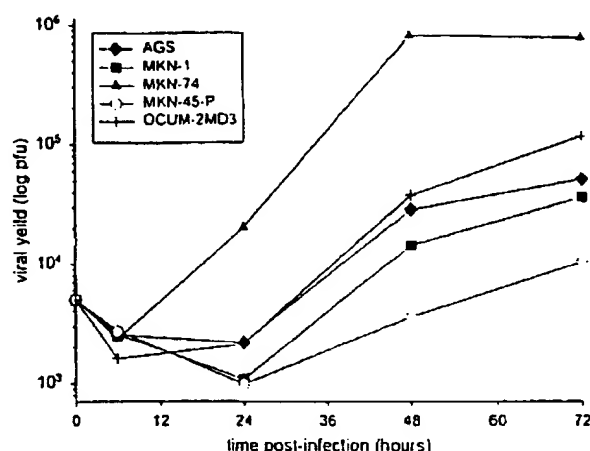


Fig. 2 G207 replication in five human gastric cancer cell lines after in vitro infection with an MOI of 0.01 (5000 pfu). Virions were collected at 6, 24, 48, and 72 h postinfection and titered by Standard Plaque assay

MOI of 0.01, with 65–87% cell death for three cell lines (OCUM-2MD3, AGS and MKN-74) and 9–52% cytotoxicity for the other two cell lines at 96 h (MKN-1 and MKN-45-P).

#### In vitro viral proliferation

To compare the ability of human gastric cancer cells to permit viral replication, cells were infected at an MOI of 0.01 with 5000 pfu, and virions were recovered and titered over 72 h (Fig. 2). All cell lines supported viral proliferation. Among the five cell lines the number of viral particles recovered within 6–24 h postinfection fell by 52–80%, suggesting viral uptake and disassembly (Fig. 2). In four of the cell lines a log phase viral burst occurred at 48 h, producing 14,000 (MKN-1), 29,000 (AGS), 38,000 (OCUM-2MD3), and 820,000 pfu (MKN-74; Fig. 2, Table 1). By 72 h the amount of virus recovered further increased in three of the cell lines to 36,000 (MKN-1), 51,000 (AGS) and 117,000 pfu (OCUM-2MD3). Infectious virions recovered for the MKN-74 cell line at 72 h reached a plateau at 790,000 pfu, secondary to significant cell death which prevented further host cell support for viral replication. The MKN-45-P cells were least supportive of viral growth, with 3600 pfu collected at 48 h, and minimal viral replication evident by 72 h, when 10,000 pfu were

collected (Fig. 2, Table 1). Further demonstrated is the relationship among these five cell lines with respect to viral replication and cytotoxicity (Table 1). Cell lines that were most supportive of viral replication were also more susceptible to the tumoricidal effects of G207

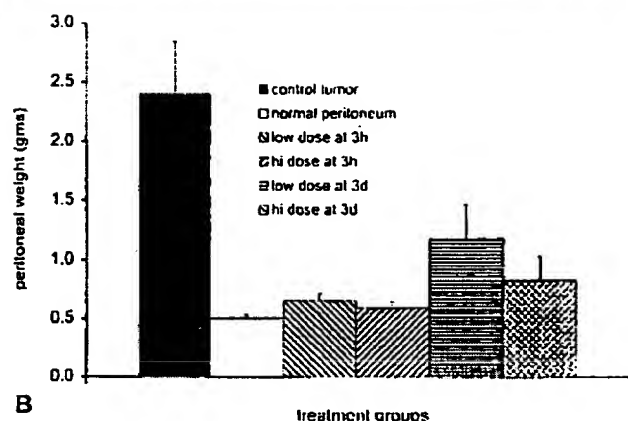
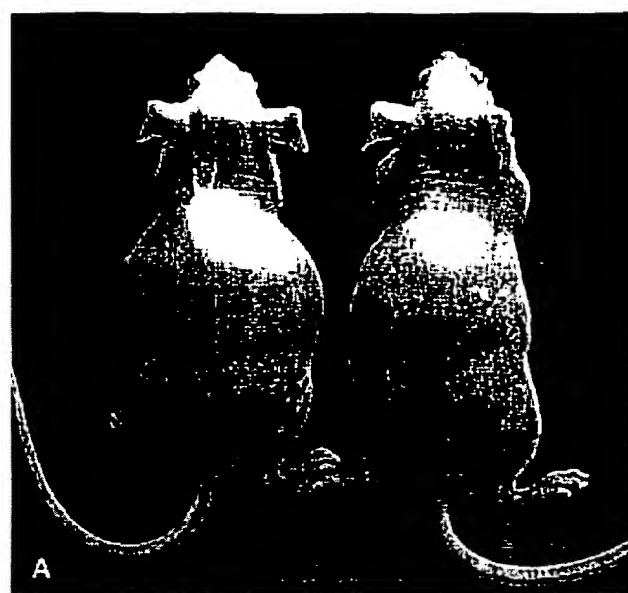
#### Treatment of gastric carcinomatosis with peritoneal G207

The ability of G207 to treat peritoneally disseminated gastric cancer was evaluated in a well established animal model [24]. All animals ( $n=50$ ) were injected i.p. with ( $2 \times 10^6$ ) OCUM-2MD3 human gastric cancer cells. To assess the ability of G207 to kill nonimplanted tumor cells, i.p. injection of  $5 \times 10^6$  or  $5 \times 10^7$  pfu of G207 (low or high dose) was delivered 3 h after tumor inoculation. The ability of G207 to suppress growth of implanted cancer cells and macroscopic tumor was evaluated by i.p. injection of  $5 \times 10^6$  or  $5 \times 10^7$  pfu of G207 3 days after tumor inoculation. At this time multiple macroscopic 1 mm nodules can be detected on the omentum and throughout the mesentery. By 3 weeks after tumor inoculation, control animals became cachectic, sluggish, and progressively distended with overwhelming tumor and bloody ascites. All control animals developed tumor, and nine of ten developed significant bloody ascites. Omental tumor size ranged between 1 and 3 cm, innumerable mesenteric nodules and gonadal fat nodules grew to sizes ranging from several millimeters to 1 cm, and the inferior diaphragm became inundated with tumor. As a group, G207-treated animals maintained or gained weight, continued to feed and groom normally, and only 4 of 39 animals developed bloody ascites, all from the low-dose, established tumor group (Table 2, Fig. 3A). At killing the tumor burden was assessed by harvesting the peritoneum with attached tumor from each animal. Omental tumor was collected as a large mass, while diaphragm, mesentery, and gonadal fat were stripped from the bowel and associated organs and weighed. Mean peritoneal weight ( $\pm$ SEM) for the control group was  $2.4 \pm 1.4$  g. Normal peritoneum was also harvested from mice ( $n=9$ ) that had never been inoculated with either tumor or virus; mean baseline peritoneal weight ( $\pm$ SEM) was  $0.50 \pm 0.09$  g. The most significant reduction in tumor burden was seen in the animals treated at 3 h. Animals treated with  $5 \times 10^6$  pfu ( $n=10$ ) had a mean peritoneal weight ( $\pm$ SEM) of  $0.65 \pm 0.19$  g ( $P=0.001$  vs. controls), and those treated with  $5 \times 10^7$  pfu ( $n=10$ ) had a mean peritoneal weight of  $0.59 \pm 0.17$  g ( $P<0.001$  vs. controls; Fig. 3B). Delivery of

Table 2 Presence of bloody ascites and cachexia in control animals vs. G207-treated animals at time of killing (day 23)

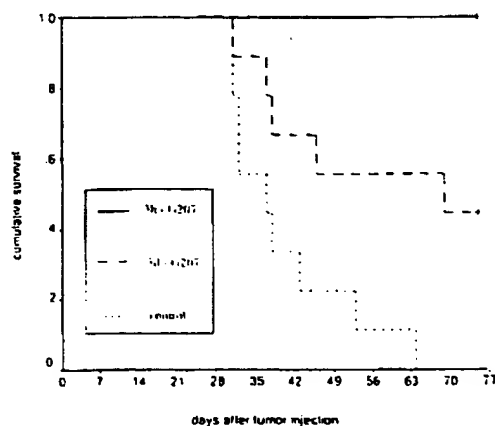
Viral dose (pfu)	Treatment time	No. of animals with bloody ascites	Weight change over 23 days (g)
Control (media)	3 days	9/10	$-5.1 \pm 4.1$
$5 \times 10^6$ (low dose)	3 days	4/10	$-3.1 \pm 2.7$
$5 \times 10^6$ (low dose)	3 h	0/10	$+1.0 \pm 1.7^*$
$5 \times 10^7$ (high dose)	3 days	0/10	$+0.37 \pm 3.3^{**}$
$5 \times 10^7$ (high dose)	3 h	0/10	$-0.27 \pm 2.6^{**}$

\* $P<0.0005$ , \*\* $P<0.01$ , unpaired Student's *t* test



**Fig. 3A, B** Efficacy of gastric carcinomatosis treatment with intraperitoneal G207. Gastric carcinomatosis was established in athymic mice by i.p. injection of  $2 \times 10^6$  OCUM-2MD3 human gastric cancer cells. Viral treatment ( $n=10$ /group) included i.p. injection of  $5 \times 10^6$  pfu (low-dose) or  $5 \times 10^7$  pfu (high-dose) G207, either 3 h or 3 days after tumor challenge; control animals were treated with media. Peritoneal tumor weights were compared between all groups. **A** A representative control mouse (*left*) demonstrates abdominal distention, bloody ascites, cachexia, and extensive tumor burden, as compared to a mouse (*right*) treated with  $5 \times 10^7$  pfu G207 at day 3. **B** A substantial reduction in mean tumor burden was seen between the G207-treated groups and the control group ( $P<0.001$  for both 3 h groups;  $P<0.05$  for the low-dose, 3-day group;  $P<0.01$  for the high-dose, 3-day group; significance was determined by unpaired *t* test)

G207 at day 3 also significantly inhibited tumor growth. The group treated with  $5 \times 10^6$  pfu at day 3 ( $n=10$ ) had a mean peritoneal weight of  $1.2 \pm 0.9$  g ( $P<0.05$ ), and those treated with  $5 \times 10^7$  pfu ( $n=9$ ) had a mean peritoneal weight of  $0.82 \pm 0.61$  g ( $P<0.01$ ; Fig. 3B). The peritoneal weights of animals treated with  $5 \times 10^7$  pfu of G207 after 3 h or 3 days showed no statistically significant difference to the normal peritoneum ( $P>0.1$ ). One animal in



**Fig. 4** Extended survival after intraperitoneal G207 treatment of gastric carcinomatosis. Mice were injected with  $2 \times 10^6$  human gastric cancer cells and treated with i.p. G207 ( $5 \times 10^7$  pfu) either 3 h or 3 days later ( $n=9$ /group); control animals were treated with media ( $n=9$ ). All control animals died, with 100% survival (9/9) in the 3-h treatment group ( $P<0.0001$ ), and 44% survival (4/9) in the 3-day treatment group ( $P=0.01$ ) at 77 days. There was also a significant difference between G207 treatment at 3 h and 3 days ( $P=0.01$ ). Kaplan-Meier test was used for statistical analyses

the high-dose, 3-day treated group died from complications during i.p. injection. All groups were statistically compared using a two-tailed, unpaired *t* test.

#### Survival study after peritoneal G207 treatment of gastric carcinomatosis

Mice were treated with  $5 \times 10^7$  pfu of G207 either 3 h ( $n=9$ ) or 3 days ( $n=9$ ) after tumor inoculation. Control animals demonstrated 100% mortality by 64 days, with a mean survival time of 40 days. The animals treated after 3 days showed significantly prolonged survival, with four of nine mice (44%) alive at day 77, and a mean survival of 58 days ( $P=0.01$ , Fig. 4). Treatment of animals after 3 h resulted in 100% survival at day 77 ( $P<0.0001$ , Fig. 4). There was also a statistically significant difference between the 3-h and 3-day G207 treatment groups ( $P=0.01$ ). The Kaplan-Meier test was used for statistical analyses.

#### Histochemical analysis of the extent of G207 infection and dissemination in vivo

The extent of G207 infection and dissemination was examined after i.p. delivery by staining for the marker gene product  $\beta$ -galactosidase. Tissue containing this *lacZ* gene product stains blue in the presence of X-gal solution. Organs and tumors were collected 1, 3, 7, 12, and 20 days after i.p. G207 delivery. No blue cells were observed in organs (brain, liver, heart, lungs, spleen, bowel, bladder, testicle, kidney) evaluated at these time points. Tumor specimens showed blue staining on days 1 and 3 only,

with no staining seen 7, 12, or 20 postinfection. The staining at day 1 showed blue cells at the periphery of tumor, while day 3 staining showed blue cells closer to the center of the tumor specimen (picture not shown).

Quantification of peritoneal viral clearance, viral dissemination, and viral proliferation of G207 and wild-type KOS virus in vivo

Results from real-time quantitative PCR analysis demonstrated that G207 replication selectively occurred within tumor specimens in vivo. An evaluation of peritoneal viral clearance after i.p. delivery was also performed. The amount of viral genome present in DNA extracted from tumor, brain, liver, bowel, kidney, and lavage fluid was assessed by quantitative PCR using a HSV *ICP0* gene specific primer and probe set (PE Applied Biosystems). Use of a standard curve determined the limit of reliable viral detection to be one viral particle per 10 ng genomic DNA. Both G207 and KOS virus replicated within tumor specimens in vivo. PCR analysis of peritoneal tumor specimens from KOS-treated animals detected a mean of  $4.2 \times 10^5$  viral genomic equivalents on day 1 (in 1  $\mu$ g genomic DNA), with an increase to  $2.9 \times 10^6$  viral genomes by day 2, and a peak of  $3.5 \times 10^6$  genomes by day 3, representing an 8-fold increase between days 1 and 3 (Fig. 5A). The amount of KOS virus detected at days 5 and 7 decreased to  $2.1 \times 10^6$  and  $8.6 \times 10^5$  viral genomic equivalents, respectively (Fig. 5A). KOS-treated animals demonstrated signs of CNS toxicity by day 7 and were sluggish and failed to groom properly. These animals were killed, and no time points were collected after day 7. Analysis of brain tissue demonstrated quantities of virus reaching  $3.3 \times 10^6$  viral equivalents (in 1  $\mu$ g genomic DNA) in the sickest mouse. PCR data from G207-treated animals detected a mean of 1200 viral genomic equivalents (in 1  $\mu$ g genomic DNA) on day 1, with a 7-fold increase to 9300 viral genomes by day 2 (Fig. 5A, B), and a 62-fold increase to 74,500 viral genomes by day 3 (Fig. 5B). This number decreased to 22,000 and 1600 viral genomic equivalents by days 5 and 7, respectively, and remained below 100 viral genomes through day 20 (Fig. 5B). Peritoneal lavage fluid obtained from animals immediately after i.p. injection of G207 (day 0) contained significant viral DNA, with a mean of 130,000 viral genomic equivalents in 10 cc lavage fluid (Fig. 5B). Rapid viral clearance was noted by day 1, when 2200 viral genomes were detected in 10 cc of lavage fluid (Fig. 5B). No viral genomes were found in peritoneal lavage fluid on days 3, 7, and 12 (Fig. 5B). To evaluate dissemination of G207, several organs were analyzed by quantitative PCR. Brain tissue contained 1050 viral genomes detected on day 1, which decreased and remained persistent at 500 viral genomes detected through day 20. Viral genomic equivalents were not found within kidney specimens until day 20, when a mean of 9400 viral genomes were detected. Liver and bowel specimens did not contain any viral genomes within the limits of re-

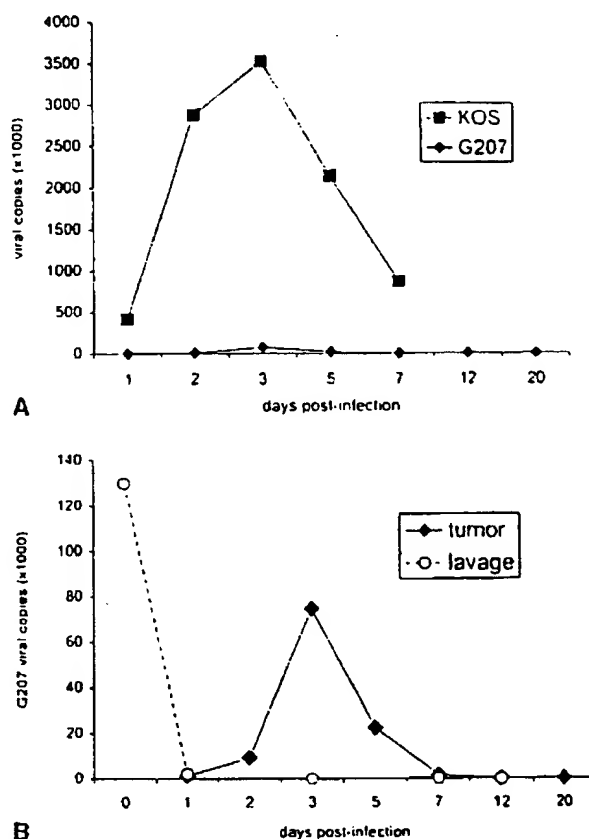


Fig. 5A, B G207 viral proliferation selectively occurs within tumor cells in vivo. Athymic mice injected i.p. with human gastric cancer cells ( $2 \times 10^6$ ) were treated 3 days later by peritoneal delivery of  $5 \times 10^7$  pfu of either wild-type KOS virus or the multimutant virus G207. Tumor specimens were harvested at multiple time points postinfection. Quantitative PCR was used to amplify the herpes *ICP0* gene in all specimens. A The wild-type KOS virus replicated within tumor specimens significantly, with peak proliferation 3 days after infection. Animals were killed 7 days postinfection secondary to significant neurotoxicity. B Peritoneal lavage fluid was collected in addition to tumor specimens from animals treated with G207. The majority of viral genomic equivalents were cleared from the peritoneum within 1 day, with no detectable viral genomes isolated thereafter. Viral uptake by peritoneal tumor was seen within 1 day after i.p. delivery of G207. A 62-fold increase in the number of viral genomes was detected by day 3, indicative of viral proliferation in vivo. The number of viral genomes in tumor specimens diminished considerably by day 7, with few detectable copies of the *ICP0* gene by day 20 (lavage fluid not tested on day 20).

liable detection based on the standard curve. Analysis of tumor, brain, kidney, and liver was performed on one mouse that lasted 100 days in the survival experiment, with no viral genomes detected within the limits based on the standard curve.

## Discussion

G207 is a multimutated, attenuated herpes simplex virus that has shown promise in treating both neurological and



nonneurological malignancies, with replication restricted to tumor cells [13, 14, 17]. The ability of this virus selectively to kill tumor cells is based upon strategic mutations. G207 contains deletions of both copies of the  $\gamma_134.5$  gene, which results in attenuated neurovirulence and poor reactivation from latency [13, 19, 20]. This viral gene has also been shown to prevent some cells from undergoing apoptosis, the normal cellular response to HSV infection [25, 26]. Cells infected with  $\gamma_134.5$  mutants may be signaled to continue with programmed cell death. G207 also contains a *lacZ* insertion mutation which inactivates ribonucleotide reductase, an enzyme required for synthesis of both viral and cellular DNA [13, 21]. In contrast to nondividing cells, tumor cells have been shown to express high levels of ribonucleotide reductase, which complements this deletion and supports viral replication [15, 21, 22, 27]. This is one mechanism for selective replication of G207 within tumor cells, sparing normal tissue. The current report demonstrates that regional delivery of this oncolytic virus is effective treatment for peritoneally disseminated malignancy. G207 can be used to kill five human gastric cancer cell lines in vitro, and susceptibility to viral replication is correlated with cytotoxicity. Peritoneal viral delivery inhibits tumor growth and prolongs survival in an animal model of gastric carcinomatosis. Reduction in tumor burden from G207 treatment also inhibits cachexia. Results from quantitative PCR analysis reveal the presence of in vivo viral replication within tumor cells, which is correlated with the pattern of viral proliferation observed in vitro. Finally, evaluation of wild-type KOS virus demonstrates significant in vivo replication as well, albeit with significant CNS toxicity.

The overall cell kill produced by replication-competent viruses depends on both the direct effects of the administered virus and the effects of progeny viruses produced by infected tumor cells [13, 18, 23, 28]. Using an MOI of 1, the inherent cytotoxicity of G207 to kill five human gastric cancer cell lines is demonstrated in vitro, with greater than 96% cell kill seen by 96 h. Significant cell death is also demonstrated at an MOI of 0.1 and 0.01, where viral replication and secondary infection is necessary for cytotoxicity to occur. Viral growth curves were performed at an MOI of 0.01 to show a significant difference among these five cell lines with respect to viral proliferative capacity. An MOI of 0.01 was used to prevent the significant cell death seen at higher MOIs, which would destroy cellular substrate before viral replication could be measured. Cell death was correlated directly with the degree of viral proliferation. These results are important confirmation that viral proliferation may be a major determinant of the tumoricidal actions of oncolytic viruses. Evaluation of tumor cell permissiveness to viral replication may provide clinical usefulness in determining which tumors are potentially most susceptible to viral cytotoxicity after oncolytic therapy.

Other studies have attempted to demonstrate the effectiveness of oncolytic viruses by direct injection into established CNS and colorectal tumor implants [13, 14,

23, 27, 29]. However, this does not simulate the clinical situation since most tumors amenable to therapy by direct injection can be surgically excised. Regional vascular infusion has also shown promise in delivering oncolytic viruses to treat malignancy confined to the liver, using a clinically relevant model [15, 30]. Our investigation illustrates the potential of localized oncolytic viral therapy in treating disseminated peritoneal malignancy. The ability of G207 to kill human gastric cancer cells in vivo is demonstrated by the reduction in tumor burden and prolongation in survival in an animal model of gastric carcinomatosis. Peritoneal disease occurs over a large surface area, yet remains confined to a single compartment. There are sound theoretical grounds for using oncolytic viral therapy to treat a localized malignancy. Regional delivery may limit the number of viral particles necessary for tumor kill and thereby minimize toxicity. Increased concentrations of virus at the site of disease may also improve exposure of virus to tumor. Kucharczuk et al. [18] demonstrated that the oncolytic agent HSV-1716 can kill a peritoneal mesothelioma cell line in vitro and in vivo. The studies in this report evaluate viral kinetics and tumoricidal effect after peritoneal viral delivery in a model of gastric carcinomatosis. This model simulates a clinically relevant scenario, as gastric cancer is common worldwide, with carcinomatosis accounting for much of the mortality seen in this disease [3, 31].

Gastric carcinomatosis occurs when free intraperitoneal cancer cells implant on the surface of the peritoneum. Cancer cells exfoliate into the peritoneum naturally after tumors invade through the gastric serosa, and iatrogenically during surgical manipulation. These exfoliated cancer cells are viable and capable of implanting and proliferating within the peritoneum [32, 33]. The presence of free intraperitoneal cancer cells is an independent predictor of poor outcome and is responsible for the high rate of isolated peritoneal metastatic recurrence seen in gastric cancer [34, 35]. Peritoneal chemotherapy has been used perioperatively to kill free cancer cells, albeit with minor benefit and significant morbidity [6, 7, 8]. Results from this investigation demonstrate the efficacy of using oncolytic viral therapy in the treatment of free intraperitoneal cells, which is a necessary target if peritoneal dissemination is to be prevented. These data also demonstrate successful treatment of macroscopic tumor nodules when peritoneal dissemination is already established.

Several factors need to be taken into consideration with respect to the potential clinical applications of regional oncolytic therapy. The OCUM-2MD3 cell line is one of the most sensitive cell lines of five evaluated in this investigation, which may account at least in part, for the significant efficacy seen after i.p. G207 therapy. Not all human tumors can be expected to demonstrate such susceptibility to viral replication and tumor kill, and some may be totally resistant to the effects of viral therapy. It may also be difficult to treat human tumors just hours after free cancer cells are present within the perito-



neum, which resulted in the best antitumor effects in this study. The strategy of perioperative, intraperitoneal chemotherapy does indeed attempt to eliminate this microscopic residual disease [3, 36]. It is not unreasonable to use peritoneal oncolytic viral therapy in this fashion. Many patients with gastric cancer, however, have advanced disease at the time of initial diagnosis [5, 37]. Future applications of oncolytic viral therapy may therefore require the use of less attenuated viruses, and should also evaluate various combination therapies. Indeed, these studies are currently being performed, and the development of new multimutant HSV vectors have shown excellent tumor targeting with oncolytic efficacy comparable to that of wild-type virus, in addition to the maintenance of neuroattenuation [38]. Combinations between viral therapy, chemotherapy and cytokine therapy have also been shown to work better than either agent alone [39, 40]. These new developments are especially promising for tumors that may be partially resistant to single-agent therapy. This study also shows that the development of ascites and cachexia can be reduced or eliminated by treating carcinomatosis with G207. Palliative treatment for patients with advanced gastric cancer using oncolytic therapy is promising for those who cannot be cured.

Real-time quantitative PCR was used to determine viral presence and proliferation in various organs after both G207 and wild-type KOS infection. Significant quantities of KOS virus were found in brain tissue, consistent with the known natural neurotropism of the HSV. Measurable amounts of G207 were detected in brain and kidney as well, and was absent from liver and bowel. Several studies have shown that  $\gamma_134.5$  mutants are indeed capable of infecting normal neurological and epithelial tissue, although proliferation is substantially attenuated, and little virus is recovered several weeks post-infection [19, 20, 29, 41]. Cells infected with  $\gamma_134.5$  mutants undergo shutdown of total protein synthesis, which also prevents viral protein production [25, 26]. Tumor cells complement this deletion and are able to permit viral replication [12, 18, 28, 29]. In this report, quantitative data from PCR demonstrate the failure of G207 to replicate within brain tissue and other organs. Histochemical analysis for the *lacZ* marker gene demonstrates the inability of viral proteins to be expressed despite viral presence. Similarly, viral presence is detected in kidney at day 20 by PCR and may reflect viral clearance through the kidneys, yet *lacZ* expression remains negative. The absence of detectable viral genomes from brain, kidney, or liver 100 days postinfection also supports systemic G207 clearance over time. Furthermore, no systemic toxicity was evident in mice treated with G207, while mice treated with KOS died from CNS injury.

Quantitative PCR was also used to evaluate the clearance of virus from the peritoneum and to demonstrate in vivo viral proliferation. The pattern of viral uptake and proliferation seen among five gastric cancer cell lines in vitro parallels the situation observed in our in vivo ex-

periments. Peritoneal clearance of G207 is rapid, with viral uptake detected in implanted tumor cells 1 day after peritoneal delivery of virus. Over 3 days, although all virus has been cleared from the peritoneum, there is a peak 62-fold increase in the number of G207 viral genomic equivalents present in tumor. These results signify viral replication in peritoneal tumor cells in vivo, which occurs in both G207 and KOS viruses. The difference between KOS and G207 replication is that it is 47-fold greater for KOS, which is expected from the unattenuated wild-type virus. As tumor is killed by viral lysis, continued viral production cannot occur with the loss of tumor substrate, resulting in a decrease in viral genomes detected from days 5, 7, 12, and 20. The small amount of *ICP0* gene copies present at the later time points is presumably from residual tumor harboring viral genes. In an animal that survived to 100 days neither tumor nor virus was detected in the peritoneum, as complete viral eradication of tumor occurred. While others have documented proliferation of oncolytic viruses using in vitro assays, the current data verify such proliferation in vivo [12, 13, 18, 23]. These data indicate that in vitro assays may be used to predict in vivo viral behavior. The presence of viral production in vivo may allow biological oncolytic activity to occur after delivery of virus at MOIs well below the ultimate effective dose.

G207 is capable of proliferating within experimental human gastric cancer cells in vitro and in vivo. Viral growth curves may be useful in screening tumors that can support viral replication, resulting in tumor cell lysis and tumor killing. These data encourage clinical trials involving G207 not only for gastric cancer but for other peritoneal malignancies.

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